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(11) Publication number:

**0 139 201  
A1**

(12)

# EUROPEAN PATENT APPLICATION

(21) Application number: 84110703.0

(51) Int. Cl.<sup>4</sup>: G 01 N 33/534

G 01 N 33/53, G 01 N 33/68

C 07 K 7/06, C 07 K 5/10

(22) Date of filing: 07.09.84

(30) Priority: 09.09.83 GB 8324167

(43) Date of publication of application:  
02.05.85 Bulletin 85/18

(84) Designated Contracting States:  
DE FR GB IT SE

(71) Applicant: THE WELLCOME FOUNDATION LIMITED  
183-193 Euston Road  
London NW1 2BP(GB)

(72) Inventor: Chang, Kwen-Jen  
2476 Foxwood Drive  
Chapel Hill North Carolina 27514(US)

(74) Representative: Sandmair, Kurt, Dr. Dr. et al,  
Patentanwälte Dr. Berg Dipl.-Ing. Stapf Dipl.-Ing.  
Schwabe Dr. Dr. Sandmair Postfach 86 02 45  
Stuntzstrasse 16  
D-8000 München 86(DE)

(54) Diagnostic method and kit.

(57) A method for ascertaining whether a human infant is at risk from the sudden infant death syndrome comprising the steps of

a) measuring the concentration in a blood sample from the infant of at least one of morphiceptin and  $\beta$ -casomorphin; and

b) determining the risk to the infant using the blood concentration measurement(s).

Also provided are labelled morphiceptin, labelled  $\beta$ -casomorphin and a diagnostic kit of value for the performance of the said method.

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DIAGNOSTIC METHOD AND KIT

The present invention relates to a method for the identification of human infants at risk from the sudden infant death syndrome, and to compounds and a diagnostic kit of value for the performance thereof.

The sudden infant death syndrome (SIDS), alternatively called cot death or crib death, has been defined as "the sudden death of any infant or young child which is unexpected by history and in which a thorough post mortem examination fails to demonstrate an adequate cause for death". It is recognized as a distinct clinical entity that results in about 1500 deaths a year in the United Kingdom and 8000 deaths a year in the United States of America: in one study the syndrome accounted for 45% of postperinatal infant deaths and 20% of all deaths from 8 days to 14 years of age (for reviews see Valdes-Dapena, M.A.: Pediatrics 66: 597, 1980 and Guntheroth, W.G.: Am. Heart J. 93: 784, 1977).

Despite increasing research into the syndrome its pathogenesis remains obscure and there is no satisfactory method for the identification (prediction) of infants at risk. The problem at hand is identified thus by Valdes-Dapena (loc. cit.):

"Ideally, the physician should be able to determine, upon the basis of physical examinations and certain tests, whether or not a newborn baby is apt to die of sudden infant death syndrome. Unfortunately, although some progress has been made toward that end within the past decade, that day has not yet arrived . . . Thus, at this time, there is no reliable screening mechanism

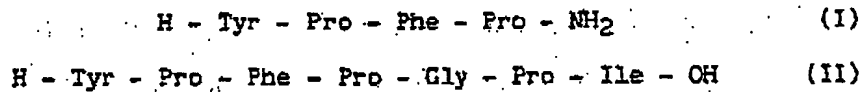
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-2-

0139201  
8408

for sudden infant death syndrome; it remains an entity, the occurrence of which, for any specific infant, cannot be predicted with any degree of certainty".

The present invention provides a method for the identification of such at-risk infants based on a determination of the level (concentration) in their body fluids, preferably blood, of either or both of the peptides morphiceptin (I) and 6-casomorphin (II)



(all chiral amino acid radicals are in the L-configuration).

It is envisaged that such a determination procedure will form a part of the routine perinatal assessment of all infants with those shown to have an abnormally high blood level of either or both of the said peptides being regarded as at risk from the syndrome. There is therefore enabled the possibility of monitoring thus-selected infants in an attempt to avert their falling victim to SIDS.

In one aspect therefore there is now provided a method for ascertaining whether a human infant is at risk from the sudden infant death syndrome comprising the steps of

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-3-

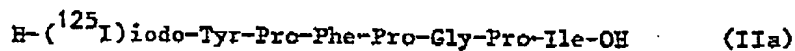
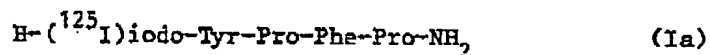
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- a) measuring the concentration in a blood sample from the infant of at least one of morphiceptin and  $\beta$ -casomorphin; and
- b) determining the risk to the infant using the blood concentration measurement(s).

In a preferred aspect the concentration of  $\beta$ -casomorphin is measured.

Measurement of the peptide concentration(s) in the blood sample (step(a)) may be effected using any conventional technique however, for general convenience, immunoassay is preferred. When an immunoassay procedure is employed the known peptide(s) may be labelled in any manner enabling determination of the amount bound; radiolabelling (radioimmunoassay) is particularly convenient using for example a compound labelled with  $^3\text{H}$  or, preferably, a  $\gamma$ -emitting isotope such as  $^{125}\text{I}$ . Conveniently the assay is carried out upon a morphiceptin/ $\beta$ -casomorphin-containing fraction prepared from the blood sample as originally withdrawn, for example plasma, serum or a fraction thereof.

Morphiceptin and  $\beta$ -casomorphin may be labelled with  $^{125}\text{I}$  by the chloramine T method standard in the art to provide respectively the compounds (Ia) and (IIa)



A diagnostic kit of value for the performance of the method described herein comprises

B408

0139201

- 4 -
- a) at least one of morphiceptin and  $\beta$ -casomorphin labelled in a manner enabling determination of the amount thereof bound in an immunoassay procedure,
  - b) (complementary to a)) at least one of antibodies against morphiceptin and antibodies against  $\beta$ -casomorphin, and
  - c) (complementary to a)) a standard of at least one of morphiceptin and  $\beta$ -casomorphin,

the materials being held in separate sealed vessels.

In a preferred aspect such a kit comprises, as a), at least one of  $^{125}\text{I}$ -labelled morphiceptin and  $^{125}\text{I}$ -labelled  $\beta$ -casomorphin; in a more preferred aspect a) is  $^{125}\text{I}$ -labelled  $\beta$ -casomorphin.

Conveniently the said kit also comprises at least one of

- d) a sealed vessel containing a buffer solution, and
- e) a sealed vessel containing a protease inhibitor, for example bacitracin.

One or more of the above-indicated materials may, if desired, be held in a freeze-dried (lyophilized) condition until required for use.

-5-

0139201

8408

Blood sample collection and fractionation

1. Draw 1 mL blood into tubes containing EDTA
2. Place the tubes in a 4°C ice bucket
3. Centrifuge to separate the cells from the plasma
4. Mix 0.5 mL plasma with 0.5 mL cold 10% trichloroacetic acid
5. Centrifuge to separate the resulting precipitate from the supernatant
6. Prepare a reverse-phase C-18 Sep-Pack cartridge; wet the cartridge with methanol, then 0.1 M acetic acid
7. Apply the supernatant from 5. to the prewetted C-18 Sep-pack cartridge
8. Wash the cartridge with 30 mL 1 M acetic acid
9. Elute peptides from the cartridge with 3 mL acetic acid-methanol (1:17)
10. Dry the eluate under nitrogen
11. Lyophilize the residue
12. Dissolve the residue in 0.20 M phosphate buffer (pH 7.4)
13. Assay for morphiceptin-like and  $\beta$ -casomorphin-like activities

Radioimmunoassay for morphiceptin-like and  $\beta$ -casomorphin-like activities

1. Synthetic morphiceptin and  $\beta$ -casomorphin were prepared using solid-phase techniques.
2. Antisera against morphiceptin and  $\beta$ -casomorphin were raised in male New Zealand white rabbits. The respective peptide was conjugated to bovine serum albumin (Pentex 5 x crystallized) by 1% glutaraldehyde in 0.20 M sodium phosphate buffer (pH 7.4) for 15 min. at room temperature. The conjugate was

SP3/sp/4

0139201

-6-

8408

separated from unreacted peptide by gel filtration chromatography on a Sephadex G-25 column. The void volume fraction containing conjugate (200  $\mu$ g peptide) was then emulsified with an equal volume of Freund's complete adjuvant and injected into rabbits subcutaneously on both sides of the body and intramuscularly on both hind paws. The rabbits were boosted every three weeks with the conjugate (about 50  $\mu$ g peptide) emulsified with Freund's incomplete adjuvant.

3. Radioimmunoassay (RIA) using protein A-containing *Staphylococcus* as solid phase support (ref. 1) was employed. Specific antiserum (0.5 mL) was incubated with washed *Staphylococcus* in a 50 mM Tris. HCl buffer (pH 7.7) containing 0.5 mM EDTA and 0.5% Triton X-100 for 3 hrs. to absorb most IgG. The IgG-absorbed *Staphylococcus* was then washed twice with the same buffer. The final pellet was suspended in 5 times the original serum volume and stored at 4°C.

4.  $^{125}$ I-labelled morphiceptin and B-casomorphin were prepared by the chloramine T method (ref. 2). An aliquot (5-10  $\mu$ L, 3 mCi) of carrier free and freshly prepared Na  $^{125}$ I (Union Carbide) was added to 100  $\mu$ L of 0.25 M sodium phosphate buffer (pH 7.4) containing 1.5 nmoles of peptide. The iodination was initiated by adding 20  $\mu$ L of 0.5 mg/mL Chloramine T and terminated 20 seconds later by adding 20  $\mu$ L of 1 mg/mL sodium metabisulfite. 100  $\mu$ L of 0.1% bovine serum albumin containing Tris. HCl (50 mM) buffer was added 30 sec. later. The whole reaction mixture was applied to Biol-Gel-P-2 (12 mL) pre-equilibrated with Tris. HCl - 0.1% bovine serum albumin buffer

-7-

0139201  
8408

solution. The iodinated peptide peaks (8-9 mL fractions for  $\beta$ -casomorphin, 10-11 mL fractions for morphiceptin) were collected.

5. RIA was carried out in 0.20 mL of sodium phosphate buffer (pH 7.4) which is 0.5 mM with respect to EDTA and contains 1% Triton X-100 for 18 hrs. at 4°C with about 10,000 cpm of  $^{125}\text{I}$ -labelled peptide. At the end of the incubation, 1 mL of the same cold buffer was added to each tube and the mixture centrifuged for 20 min. at 3500 rpm in a Sorvall RC-5 (rotor GSP-25). The supernatant was aspirated. The tubes containing *Staphylococcus* were counted in a  $\gamma$ -counter at 75% efficiency. The nonspecific binding (less than 5%) was determined by the presence of an excess of unlabelled peptide (10  $\mu\text{M}$ ).

6. The amounts of morphiceptin-like and  $\beta$ -casomorphin-like activities in unknown samples were determined from standard curves obtained with the synthetic peptides.

Ref. 1: O'Keefe, E. and Bennett, V. (1980) J. Biol. Chem. 255, 561-568

Ref. 2: Hunter, W.M., et al. (1962) Nature 194, 495-496



B408

-8-

0139201

Alternative radioimmunoassay procedureReagents:

1. Buffer. All dilutions made in 154 mM NaCl, 25 mM  $\text{Na}_2\text{HPO}_4$  pH 7.5, 0.1% Triton X-100 (trade name).
2. Bacitracin. 25 mg/ml in buffer, to give 0.5 mg/ml final concentration in the assay. Store at 4°C.
3. Solid Phase Immunoabsorbent. Donkey anti-rabbit IgG immobilized on beaded cellulose (Wellcome Reagents Anti-Rabbit Sac-Cel). Wash and resuspend to original volume in buffer.
4. Antiserum (rabbit). Prepare a working dilution of 1:200 in buffer. Final dilution in assay to be 1:10,000.
5. Radiolabel. Prepare a working concentration of radiolabel of  $5-7 \times 10^6$  cpm/ml to give approximately 35,000 cpm per assay tube.
6. Standards. Prepare stock solutions of each peptide at 1.0 mM in buffer. Store at -20°C. Prepare initial working dilutions of 1.0 (for B-casomorphin) or 2.0 (for morphiceptin) micromolar in buffer. Dilute this by factors of 3 to prepare 6 standards ranging from 5-1000 nM (10-2000) to give 0.02-5.0 pmole/tube (0.04-10.0) in the assay.
7. NSB Standard. Use 0.1 mM peptide in buffer to give 500 pmole/tube in the assay.

B408

-9-

0139201

Protocol:

1. All reagents, procedures are performed at 4°C.
2. All tubes are adjusted to a final volume of 250 microlitres in the assay.
3. Reagents 1 and 5 may be combined prior to addition for convenience, as may reagents 3 and 4.
4. The assay is initiated with the addition of the antibody-gel reagent. Incubation ranges from 3-4 hours at 4°C.
5. The assay is terminated with the addition of 4 mL of ice-cold buffer to all tubes (except TA).
6. Centrifuge samples 15 minutes at 3000 rpm in Sorval RC 3 at 4°C.
7. Aspirate and count pellets.

Condition	Buffer	Bacit.	Label	Stds	NSB	Unkn	Antib.	Gel
Total Added (TA)	-	-	5	-	-	-	-	-
Total Bound (TB)	210	5	5	-	-	-	5	25
Non-Specific (NSB)	205	5	5	-	5	-	5	25
Standard Curve	205	5	5	5	-	-	5	25
Unknowns (serum)	var	5	5	-	-	var	5	25

(all volumes are microlitres)

-10-

0139201

Sample data: (B-casomorphin)

Condition	cpm	Average	-NSB	ZB/B <sub>0</sub>	pmol/tube
Total Added	35740	35672	-	-	-
	35604				
Total Bound	2350	2376	2039	100.0	-
	2402				
Nonspecific	330	337	-	-	-
	344				
Standard (a)	560	599	262	12.8	5.0
	638				
(b)	899	923	586	28.7	1.7
	947				
(c)	1431	1462	1125	55.2	0.6
	1493				
(d)	1850	1946	1609	78.9	0.2
	2042				
(e)	2110	2261	1924	94.4	0.06
	2412				
(f)	2300	2348	2011	98.6	0.02
	2396				
Unknown (a)	1024	952	615	25.9	2.1
	879				
(b)	1650	1611	1274	53.6	0.67
	1571				
(c)	2075	2019	1682	70.8	0.32
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-15-

0139201

What we claim is

1. A method for ascertaining whether a human infant is at risk from the sudden infant death syndrome comprising the steps of
  - a) measuring the concentration in a blood sample from the infant of at least one of morphiceptin and  $\beta$ -casomorphin; and
  - b) determining the risk to the infant using the blood concentration measurement(s).
2. The method of claim 1 wherein the concentration of  $\beta$ -casomorphin is measured.
3. The method of either of claims 1 and 2 wherein the concentration is measured by immunoassay.
4. The method of either of claims 1 and 2 wherein the concentration is measured by radioimmunoassay.
5. The method of either of claims 1 and 2 wherein the concentration is measured by radioimmunoassay using a compound labelled with  $^{125}\text{I}$ .
6. The method of any of claims 1 to 5 wherein the concentration in plasma prepared from the blood sample is measured.
7. The method of any of claims 1 to 5 wherein the concentration in serum prepared from the blood sample is measured.

-2-

0139201

8. As a mercantile unit, a kit comprising

a) at least one of morphiceptin and  $\beta$ -casomorphin labelled in a manner enabling determination of the amount thereof bound in an immunoassay procedure,

b) (complementary to a)) at least one of antibodies against morphiceptin and antibodies against  $\beta$ -casomorphin, and

c) (complementary to a)) a standard of at least one of morphiceptin and  $\beta$ -casomorphin,

the materials being held in separate sealed vessels.

9. The kit of claim 8 comprising, as a), at least one of radiolabelled morphiceptin and radiolabelled  $\beta$ -casomorphin.

10. The kit of claim 8 comprising, as a), at least one of  $^{125}\text{I}$ -labelled morphiceptin and  $^{125}\text{I}$ -labelled  $\beta$ -casomorphin.

11. The kit of any of claims 8 to 10 comprising, as a), labelled  $\beta$ -casomorphin.

12. The kit of claim 8 comprising

a)  $^{125}\text{I}$ -labelled  $\beta$ -casomorphin,

b) antibodies against  $\beta$ -casomorphin, and

B408

-13-

0139201

c) a standard of  $\beta$ -casomorphin.

13. The kit of claim 8 additionally comprising a sealed vessel containing a buffer solution.

14. The kit of claim 8 additionally comprising a sealed vessel containing a protease inhibitor.

15. A compound selected from

morphiceptin labelled in a manner enabling determination of the amount thereof bound in an immunoassay procedure, and

$\beta$ -casomorphin labelled in a manner enabling determination of the amount thereof bound in an immunoassay procedure.

16. A compound selected from

H-(<sup>125</sup>I)iodo-Tyr-Pro-Phe-Pro-NH<sub>2</sub> and

H-(<sup>125</sup>I)iodo-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-OH.



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# EUROPEAN SEARCH REPORT

0139201

Application number

DOCUMENTS CONSIDERED TO BE RELEVANT			EP 84110703.0
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
A	EP - A1 0 070 478 (BYK-MALLINC-KRODT CHEMISCHE PRODUKTE GMBH) * Page 6, claims 1,5,7,8 *	1,4,5, 7,8,13	G 01 N 33/534 G 01 N 33/53 G 01 N 33/68
A	DD - A - 157 095 (NEUBERT et al.) * Claim 1 *	12,15, 16	C 07 K 7/06 C 07 K 5/10
A	US - A - 3 983 099 (G.D. NISWENDER) * Claim 1 *	12,15, 16	
A	GB - A - 2 072 192 (ABBOTT LABORATORIES) * Abstract *	12,15, 16	
A	CHEMICAL ABSTRACTS, vol. 93, no. 7, August 18, 1980, Columbus, Ohio, USA WEI E.T. et al. "Cardiuvascular effects of peptides related to the enkephalines and B-casomorphin" Abstract-no. 61 992s Life Sci. 1980, 26 (18), 1517-22	12,15, 16	TECHNICAL FIELDS SEARCHED (Int. Cl. 4) G 01 N 33/00 C 07 K 7/00 C 07 K 5/00
The present search report has been drawn up for all claims			
Place of search VIENNA		Date of completion of the search 14-12-1984	Examiner IRMLER
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons &amp; : member of the same patent family, corresponding document</p>			

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